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Proteotyping of Mammary Tissue from Transgenic and Gene Knockout Mice with Immunohistochemical Markers: a Tool To Define Developmental Lesions

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SUMMARY Through the use of transgenic and gene knockout mice, several studies have identified specific genes required for the functional development of mammary epithelium. Although histological and milk protein gene analyses can provide useful information regarding functional differentiation, they are limited in their ability to precisely define the molecular lesions. For example, mice that carry a mutation in one of the subunits of the IκB kinase, IKKα, cannot lactate despite the presence of histologically normal alveolar compartment and the expression of milk protein genes. To further define and understand such lesions on a molecular level, we sought evidence for proteins that are differentially expressed during mammary gland development with a view to generating a tissue proteotype. Using database screens and immunohistochemical analyses, we have identified three proteins that exhibit distinct profiles. Here, using mouse models as test biological systems, we demonstrate the development and application of mammary tissue proteotyping and its use in the elucidation of specific developmental lesions. We propose that the technique of proteotyping will have wide applications in the analyses of defects in other mouse models.
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THE DEVELOPMENT of mammary epithelium is mediated by the action of peptide and steroid hormones (Hennighausen and Robinson 2001; Shillingford and Hennighausen 2001). The subsequent activation of intracellular signaling pathways influences the outgrowth of the ductal tree, the proliferation and differentiation of secretory epithelial cells during pregnancy, the maintenance of milk production during lactation, and the regression of the epithelium upon withdrawal of the suckling stimulus.

Using knockout mouse models, others and we have defined an essential role for the prolactin receptor (PRLR), Jak2–signal transducer and activator of transcription 5 (Stat5) pathway in pregnancy-mediated mammary gland development (Liu et al. 1997; Ormandy et al. 1997; Brisken et al. 1999; Miyoshi et al. 2001; Shillingford et al. 2002b). A general feature of these models is a marked reduction in proliferation, which is accompanied by the persistence of ductal structures and a failure of the epithelium to develop identifiable secretory alveolar structures. Consistent with the lack of attainment of a secretory phenotype, milk protein gene expression was not detected (Miyoshi et al. 2001). We correlated the epithelial defects with high levels of the Na-K-Cl co-transporter 1 (NKCC1) protein, indicative of virgin mammary ductal epithelial cells (Miyoshi et al. 2001; Shillingford et al. 2002a), and the absence of an Na–Pi type IIb co-

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transporter (Npt2b) protein at parturition, indicative of a lack of secretory function (Miyoshi et al. 2001). In addition, we have demonstrated the use of NKCC1 and Npt2b as proteotypic markers of mammary epithelial cell identity (Miyoshi et al. 2002).

The most common techniques currently used in mouse mammary gland biology include analyses of proliferation, evaluation of histological sections, and determination of milk protein gene expression. Despite the usefulness of these methods, they are limited in their scope and application. In particular, the expression of some milk proteins is directly downstream of the PRLR–Jak2–Stat5 pathway (Liu et al. 1997), making the analyses of milk protein gene expression in respective mutant mice suboptimal as a functional readout of development. Furthermore, the methods available do not readily permit the molecular analysis of more subtle mammary gland phenotypes. For example, although mice that possess a defective nuclear factor kappa B (NFκB) pathway are not able to support their pups at parturition, they exhibit expanded alveoli, evidence of lipid droplets, and only a limited reduction in milk protein gene expression (Cao et al. 2001). Therefore, despite seemingly adequate alveolar formation and milk protein gene expression, there is a functional defect in the secretory capacity of the alveoli. Finally, the analysis of mouse models that exhibit a delay in the process of mammary gland involution (Chapman et al. 1999; Humphreys et al. 2002) are often evaluated on the basis of their histological appearance, evidence of a delay in apoptosis, and analyses of genes associated with involution and apoptosis. However, these analyses fail to address the functional properties of the epithelial compartment.

We have therefore developed a technique to which we refer as tissue proteotyping. This method uses antibodies that exhibit distinct expression profiles in different mammary epithelial cell types. Using an immunohistochemical approach and three defined antibodies, we have identified a mammary proteotype that can be used to monitor relative changes in epithelial cell type and to assess the attainment and maintenance of secretory cell function. Using mice defective in PRLR–Jak2–Stat5, NFκB, and inhibin βB signaling, and mice with a conditional deletion of the Stat3 gene, we demonstrate the use of tissue proteotyping as a viable means to assess mammary gland development. We propose that the application of this defined mammary proteotype will prove useful in the characterization of mammary epithelial defects observed in other mouse models. Furthermore, with the use of defined antibody markers we believe that the concept of proteotyping can be used in the identification and development of additional tissue proteotypes to permit cell- and tissue-specific analysis of developmental lesions.

Materials and Methods

Antibodies

The rabbit polyclonal antibody recognizing aquaporin 5 (AQP5) was purchased from Alpha Diagnostics (San Antonio, TX). The rabbit polyclonal antibodies recognizing NKCC1 and Npt2b were obtained from Dr. Jim Turner (NIDCR, NIH, Bethesda, MD) and Dr. Juerg Biber (University of Zurich, Switzerland), respectively. The mouse monoclonal antibodies (MAbs) recognizing E-cadherin and β-catenin were obtained from BD Biosciences Pharmingen (San Diego, CA) and the mouse MAbs recognizing smooth muscle actin (SMA) were obtained from Sigma (St Louis, MO). Fluorescent-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR).

Animals

All animals were treated according to animal protocols approved by ACAUC, NIH. Athymic nude (nu/nu) mice were used for all mammary gland transplantation experiments. Knockout mouse models lacking Jak2 (Neubauer et al. 1998; Shillingford et al. 2002b), Stat5 (Teglund et al. 1998; Miyoshi et al. 2001), and inhibin βB (Robinson and Hennighausen 1997), and mice with a knock-in of the IKKα locus (Cao et al. 2001) have been described previously. For immunohistochemistry and determination of the mammary proteotype throughout mammary gland development (virgin, pregnancy, lactation, and involution), mice of the C57BL/6 strain were used.

Transplantation

Transplantation of adult and embryonic mammary glands has been described previously (Miyoshi et al. 2001; Shillingford et al. 2002b). In the case of Stat5-null mice (Miyoshi et al. 2001), adult mammary epithelium was isolated from 9-week-old virgin animals. For Jak2-null transplantation experiments, embryonic mammary anlagen were isolated from day 12.5 embryos (Shillingford et al. 2002b). Eight weeks after transplantation, transplanted mammary epithelium was removed from virgin animals for analysis. Alternatively, transplanted mice were mated and transplanted epithelium was removed 1 day after the delivery of pups to assess pregnancy-mediated mammary development. Samples were processed for immunohistochemistry as described below.

Immunohistochemistry and Imaging Analysis

Routinely, small pieces of isolated mammary gland were fixed in Tellyesniczky's fixative for 5 hr at room temperature (RT), followed by dehydration and paraffin embedding. Paraffin-embedded sections (5 μm) were cleared in xylene and rehydrated through an alcohol series. Sections were immersed in boiling antigen unmasking solution (Vector Laboratories; Burlingame, CA) for 3 min, allowed to cool, and placed in PBS containing 0.05% (v/v) Tween-20 (PBST). After blocking of the sections with 3% (v/v) normal horse serum, primary antibodies were applied (SMA, 1:1000; NKCC1, 1:1000; E-cadherin, 1:200; Npt2b, 1:100; AQP5, 1:100; β-catenin, 1:200). Sections were incubated with primary antibody for 1 hr at 37C and washed in PBST. Fluorescent-conjugated secondary antibodies (1:400; Molecular

Probes) were applied to the sections for 1 hr at RT, washed in PBST, and mounted with VectaShield (Vector Laboratories). Sections were viewed under an epifluorescence equipped Zeiss Axioscop (Carl Zeiss MicroImaging; Thornwood, NY) fitted with FITC, TRITC, and FITC/TRITC filter sets. Images were captured with a Sony DKC-5000 digital camera (Sony Medical Systems; Park Ridge, NJ).

Results

Identification of Genes Preferentially Expressed in the Mammary Gland

To identify genes present primarily in cDNA libraries derived from mammary origin, we screened the mouse EST database with full-length cDNAs. Using this approach, we identified three cDNAs that exhibited preferential expression in mammary gland libraries (Table 1). Npt2b (accession no. NM_011402) was almost exclusively expressed in the cDNA libraries derived from lactating (NMLMG and Riken) mammary tissue and was absent from non-lactating (virgin) mammary tissue (NbMMG). In contrast, NKCC1 (accession no. NM_009194) and the water transporter aquaporin AQP5 (accession no. NM_009701) showed preferential expression in the mammary tumor libraries (Mam), with relatively equal expression in virgin and lactating mammary libraries, suggesting that these membrane transporters may prove useful as prognostic markers of tumor development.

Immunohistochemical Analyses of NKCC1, AQP5, and Npt2b During Normal Mammary Gland Development

Because the mammary gland contains several cell types (predominantly adipocytes, myoepithelial cells, and ductal and secretory epithelial cells), whose ratio changes throughout mammary gland development, neither Northern nor Western analyses accurately reflect their relative contribution. Furthermore, during pregnancy, mammary epithelial cells undergo proliferation and differentiation to form alveolar structures composed of secretory cells. However, no reliable markers are currently able to discriminate between ductal cells and alveolar secretory cells. Although we have previously demonstrated high levels of NKCC1 in virgin ductal epithelial cells and reduced expression during pregnancy and lactation (Miyoshi et al. 2001; Shillingford et al. 2002a) and the induction of Npt2b during late pregnancy/early lactation (Miyoshi et al. 2001), a thorough analysis of these proteins throughout mammary development, with the exception of NKCC1 (Shillingford et al. 2002a), has not been established. Therefore, we set out to determine the cell-specificity and localization of NKCC1, AQP5, and Npt2b proteins during normal mammary development using immunohistochemistry. The specificity of each of these antibodies was determined by Western blotting analysis (data not shown).

Table 1 Mouse EST database analyses[a]

Accession	EST library/No. of clones					
NbMMG	NMLMG	Riken	Mam	Total ESTs (%)		
NM_011402 (*Npt2b*)	0	20	24	1	225	20
NM_009194 (*NKCC1*)	7	6	1	35	176	28
NM_009701 (*AQP5*)	2	4	0	43	96	51

[a]Full-length cDNA sequences representing Npt2b, NKCC1, and AQP5 were screened against the mouse expressed sequence tag (EST) database. EST clones >200 bp in length and >85% identity were considered significant. NbMMg, Soares mammary gland library (virgin); NMLMG, Soares mammary gland library (lactation day 3); Riken, Riken mammary gland library (lactation day 10); Mam, 13 cDNA libraries derived from tumors of mammary gland origin as reported by the Cancer Genome Anatomy Project (CGAP).

As shown in Figure 1A, AQP5 was apparent on the apical membrane of ductal epithelial cells during virgin development. However, analyses of additional time points revealed that it was absent in epithelial cells during pregnancy (Figures 1D and 1G), at parturition (Figure 1J), and 2 days after the removal of pups (Figure 1M). Interestingly, AQP5 protein was not detected in ductal cells during pregnancy (Figures 1D and 1G), suggesting that ductal epithelial cells in the pregnant animal are inherently different from those present in virgin mammary tissue. Further determination of AQP5 protein revealed expression in terminal end buds and at all stages of the estrous cycle (data not shown). No staining was observed in the absence of the AQP5 primary antibody (data not shown). Consistent with previous observations (Shillingford et al. 2002a), high levels of NKCC1 protein were observed at the basolateral membrane of ductal epithelial cells throughout virgin development (Figure 1B). During early pregnancy, NKCC1 protein levels appeared to decrease (Figures 1E and 1H), with some ductal epithelial cells expressing much lower levels (Figure 1E, white arrowhead) than others (Figure 1E, yellow arrowhead). As pregnancy progressed, it was apparent that although ductal structures retained a few cells expressing high levels of NKCC1 protein (Figure 1H, yellow arrowhead), the majority of developing alveolar structures exhibited lower levels (Figure 1H, white arrowhead). At parturition, although high levels of NKCC1 protein were observed in the occasional cell (data not shown), the expression of NKCC1 in secretory cells was generally low (Figure 1K), a feature that persisted into involution (Figure 1N). No staining was observed in the absence of the NKCC1 primary antibody (data not shown).

Npt2b protein was first observed on the apical membrane of developing alveoli at day 15 of pregnancy (Figure 1I, yellow arrowheads) but not in ductal epithelium. Examination of later pregnancy time points established a gradual increase in the number of

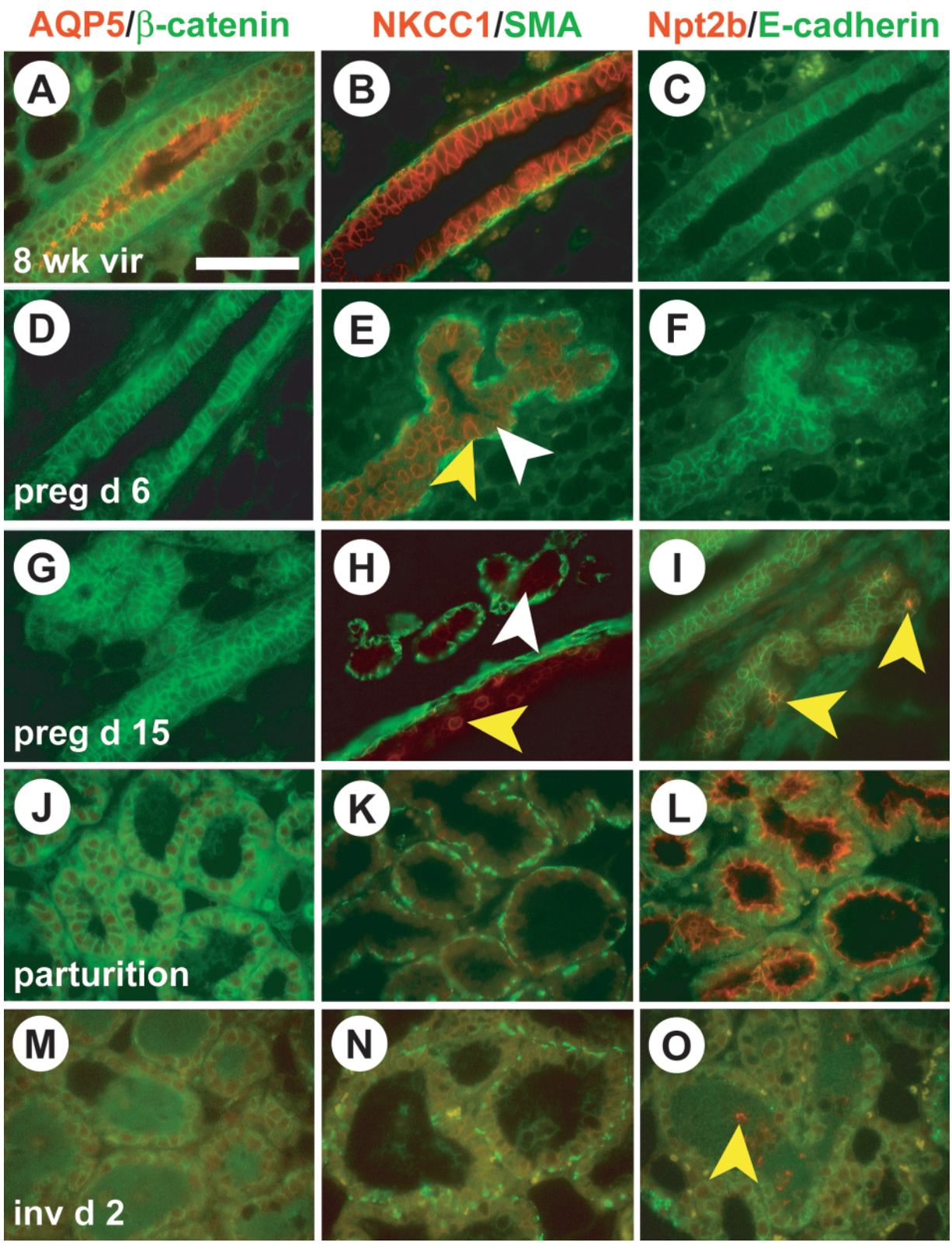

Figure 1 Localization of AQP5, NKCC1, and Npt2b during mammary gland development. Sections representing 8-week-old virgins (**A–C**), pregnancy day 6 (**D–F**), pregnancy day 15 (**G–J**), parturition (**J–L**), and involution day 2 (**M–O**) were incubated with primary antibodies directed against AQP5 (red) and β-catenin (green) (**A,D,G,J,M**), NKCC1 (red) and smooth muscle actin (green) (**B,E,H,K,N**) or Npt2b (red) and E-cadherin (green) (**C,F,I,L,O**). The localization of specifically bound primary antibodies was detected with fluorescent-conjugated secondary antibodies. Note that AQP5 was detected only in the apical membrane of the virgin (**A**) and was absent at all other time points examined. Bar = 100 μm.

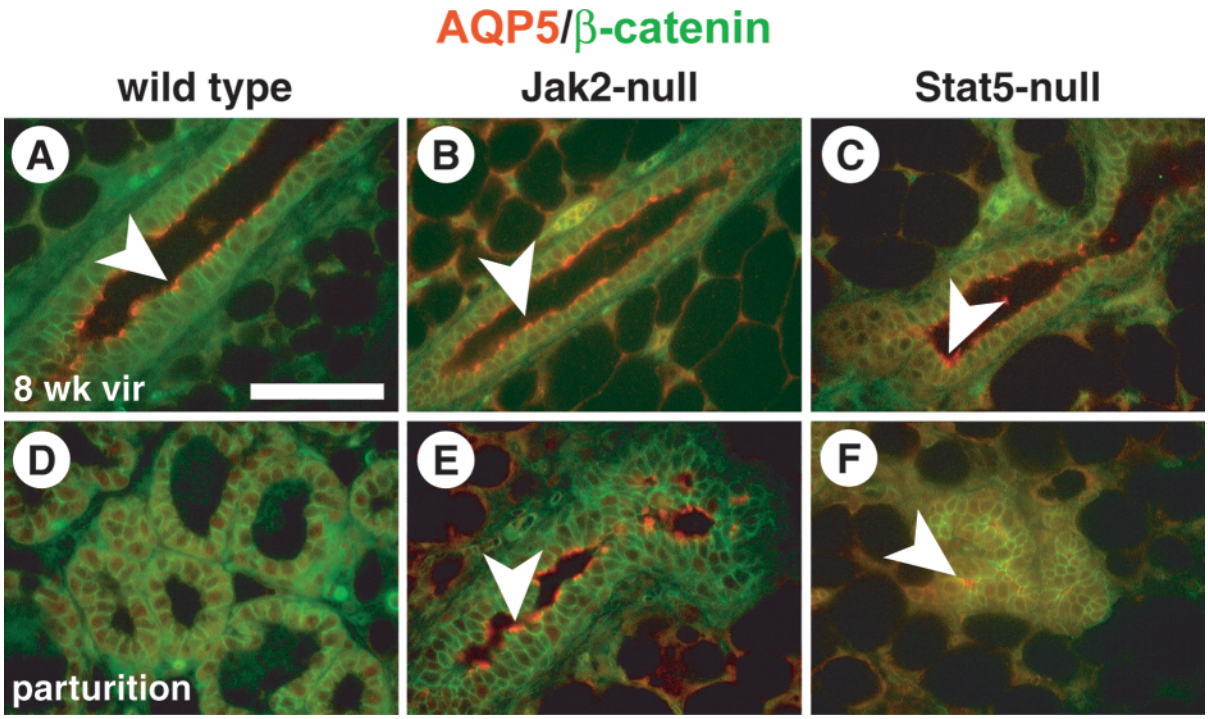

Figure 2 Persistence of AQP5 and NKCC1 in Jak2- and Stat5-null mammary epithelial cells at parturition. Sections representing 8-week-old virgins (**A–C**) and parturition (**D–F**) were incubated with antibodies directed against AQP5 (red) and β-catenin (green). The localization of specifically bound primary antibodies was detected with fluorescent-conjugated secondary antibodies. AQP5 was evident on the apical membrane (**A–C**, white arrowheads) of (**A**) wild-type, (**B**) Jak2-null, and (**C**) Stat5-null mammary ductal epithelial cells in the virgin animal. At parturition, apical AQP5 is maintained in Jak2-null (**E**) and Stat5-null (**F**) mammary cells compared to wild-type (**D**), suggestive of maintenance of ductal-like features. Bar = 100 μm.

acini with apical Npt2b protein (data not shown), which was present in all secretory cells at parturition (Figure 1L) and throughout lactation (data not shown). Interestingly, by day 2 of involution Npt2b was not detected on the apical membrane (Figure 1O), although immunoreactive protein was present in the lumen (Figure 1O, yellow arrowhead). Further experiments revealed that the disappearance of apical Npt2b protein, which occurred between days 1 and 2 of involution, was a direct result of local milk accumulation within alveolar structures and was independent of circulating hormone levels (data not shown). No staining was observed in the absence of the Npt2b primary antibody (data not shown).

Taken together, these data demonstrate that the presence of AQP5 protein and high levels of NKCC1 protein are characteristic of virgin ductal mammary epithelial cells. During pregnancy there is a significant reduction of NKCC1 protein levels in the ductal epithelium and developing alveoli which is maintained throughout lactation and early involution. In contrast, Npt2b protein is a marker of alveolar cells, as evidenced by its induction in mid-pregnancy and its continued presence throughout lactation, followed by its loss during involution.

Mammary Proteotyping of Mouse Models that Possess a Defective PRLR–Jak2–Stat5 Pathway

Using a transplantation approach, we have recently described the characterization of mammary development in the absence of the transcription factor Stat5 (both Stat5a and Stat5b) (Miyoshi et al. 2001) and the tyrosine kinase Jak2 (Shillingford et al. 2002b). These studies demonstrated that the mammary epithelium fails to undergo pregnancy-mediated cell proliferation and differentiation. As a result, alveoli fail to form, milk protein gene expression is virtually absent, and there is a persistence of adipocytes reminiscent of a virgin-like state. Analysis of NKCC1 protein revealed the maintenance of high levels of NKCC1 in Stat5- and Jak2-null mammary epithelium at parturition and the absence of Npt2b, further suggesting the presence of duct-like structures and a failure of pregnancy-mediated differentiation (Miyoshi et al. 2002; Shillingford et al. 2002b).

To extend these observations and to further understand the molecular lesions, we examined the overall pattern of AQP5 expression in these models. During virgin development, a time when the Jak–Stat pathway is believed to be inactive, AQP5 protein was evident on the apical membrane in wild-type (Figure 2A,

white arrowhead), Jak2-null (Figure 2B, white arrowhead), and Stat5-null (Figure 2C, white arrowhead) ductal epithelium. In contrast, whereas AQP5 was no longer evident on the apical membrane of mammary secretory epithelial cells in wild-type mice at parturition (Figure 2D), apical AQP5 was apparent in Jak2-null (Figure 2E, white arrowhead) and Stat5-null (Figure 2F, white arrowhead) epithelium at parturition. Taken together, the analysis of AQP5 protein in these mouse models demonstrates its usefulness as a marker of ductal epithelial cells and confirms previous data demonstrating the maintenance of a mammary ductal proteotype in postpartum mammary epithelium deficient in Stat5 (Miyoshi et al. 2001) or Jak2 (Shillingford et al. 2002b).

Proteotypic Analysis of Mammary Epithelium in Mice with Defective NFκB Signaling

The replacement in mice of serine residues by alanine residues within the activation loop of IKKα (IKKα$^{AA/AA}$) results in reduced mammary epithelial cell proliferation and differentiation, and pups born to IKKα$^{AA/AA}$ female mice fail to thrive (Cao et al. 2001). Subsequent molecular analyses revealed a mild decrease in milk protein gene expression and a marked reduction in cyclin D1 levels linked to NFκB and RANK signaling pathways. Despite these observations, many alveoli with expanded lumina were evident throughout the gland at parturition. Therefore, to gain more insight into the developmental and functional lesions observed in these mice, we examined the mammary proteotype. Analyses of NKCC1 and AQP5 protein revealed high levels of NKCC1 and AQP5 in virgin ductal epithelium and a subsequent reduction of NKCC1 during pregnancy and an absence of AQP5 similar to that observed in wild-type animals (Figure 1; and data not shown). Because IKKα$^{AA/AA}$ mice exhibit a defect during lactation, we hypothesized a lack of functional differentiation and examined the immunohistochemical localization of Npt2b protein to determine if the presence of Npt2b could be linked to alveolar function. In contrast to wild-type mice at lactation, in which all alveoli possessed apical Npt2b (Figure 3A), the majority of alveolar structures in IKKα$^{AA/AA}$ mice lacked detectable Npt2b (Figure 3B, white arrowhead) and apical Npt2b was evident in a few alveoli (Figure 3B, yellow arrowhead). Therefore, despite the manifestation of a mild mammary gland phenotype, the absence of Npt2b appears to indicate a lack of definitive alveolar function.

To address the functional consequences of a reduction in cyclin D1 levels observed in the IKKα$^{AA/AA}$ mice, an MMTV-cyclin D1 transgene was introduced. This resulted in an increase of cyclin D1 to levels seen in wild-type animals and a complete rescue of the lactational defect (Cao et al. 2001). Furthermore, milk protein gene expression levels were completely restored to wild-type levels. To determine if this apparent rescue also corresponded with the attainment of alveolar function, we examined the expression of Npt2b protein. The distribution and expression of Npt2b was not altered by transgene expression in the presence of a wild-type IKKα allele. Therefore, Npt2b was detected on the apical membrane of all alveoli in MMTV–cyclin D1 transgenic mice at lactation (Figure 3C) similar to that observed in wild-type mice (Figure 3A). However, in contrast to a lack of apical Npt2b protein in most of the alveoli present in IKKα$^{AA/AA}$ mice (Figure 3B), the introduction of the MMTV–cyclin D1 transgene and subsequent reestablishment

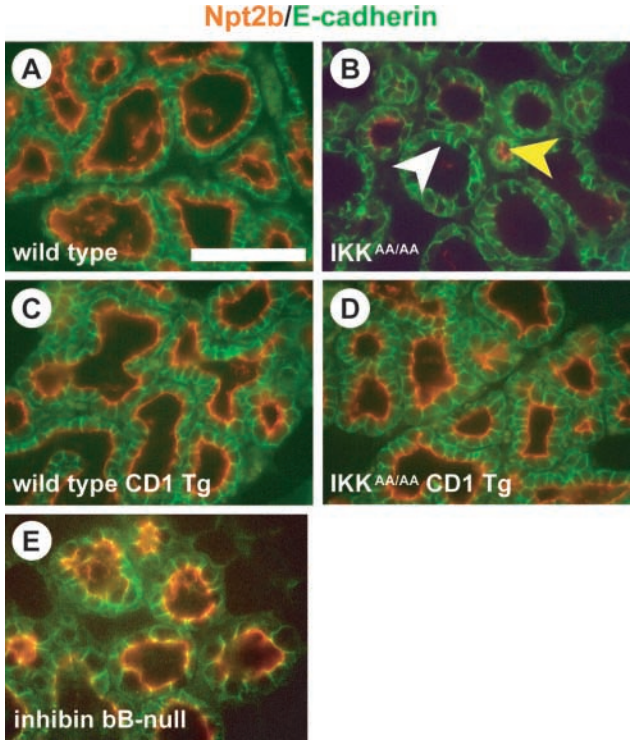

Figure 3 The absence of apical Npt2b protein in a mouse model defective in NFκB signaling is restored on restoration of cyclin D1 levels, whereas Npt2b is induced appropriately in inhibin βB-null mice. Sections representing (**A**) wild-type, (**B**) IKKα$^{AA/AA}$, (**C**) wild-type:cyclin D1 transgenic, (**D**) IKKα$^{AA/AA}$:cyclin D1 transgenic, and (**E**) inhibin βB-null mice at parturition were incubated with primary antibodies directed against Npt2b (red) and E-cadherin (green). The localization of specifically bound primary antibodies was detected with fluorescent-conjugated secondary antibodies. In the presence of a mutated IκB kinase (IKKα$^{AA/AA}$), Npt2b protein was evident only on the apical membrane of a few secretory cells at parturition (**B**, yellow arrowhead) and was absent from most alveolar structures (**B**, white arrowhead). Crossing the IKKα$^{AA/AA}$ mice into a line overexpressing cyclin D1 resulted in the restoration of cyclin D1 levels and reestablishment of apical Npt2b protein (**D**). Despite the inability of inhibin βB-null mice to lactate, Npt2b is present on the apical membrane of mammary epithelial cells at parturition (**E**). Bar = 100 μm.

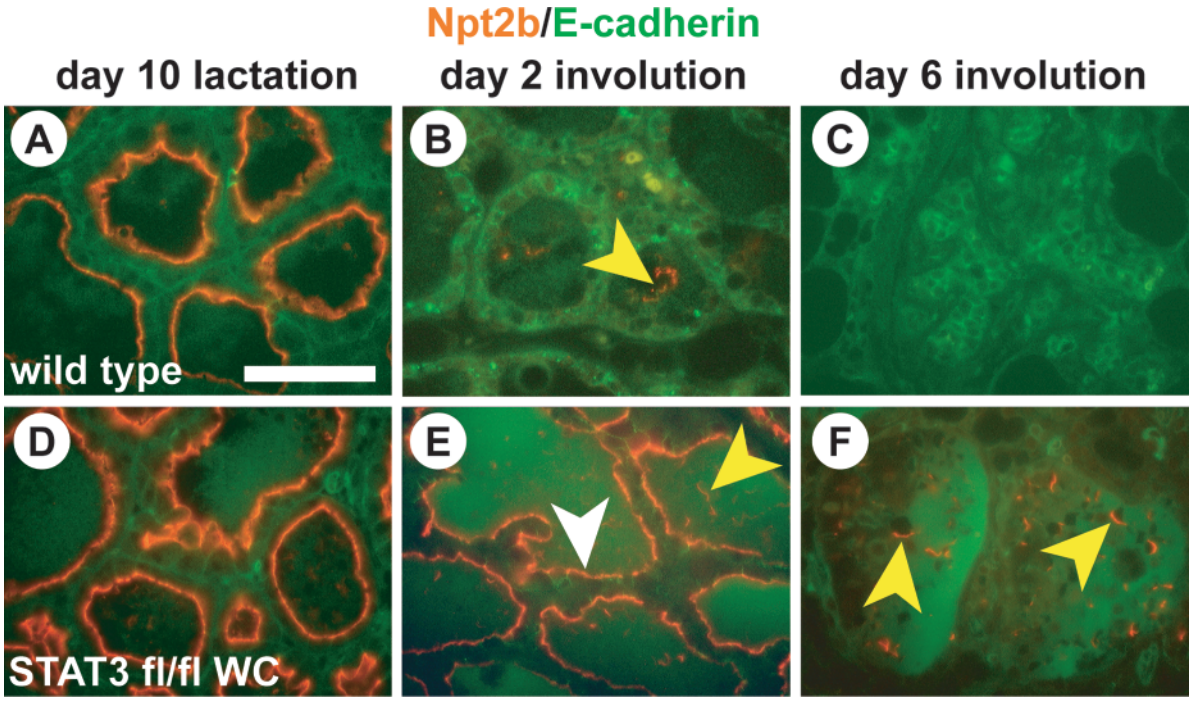

Figure 4 Delayed mammary gland involution in Stat3 fl/fl;WC mice is associated with persistence of apical Npt2b protein. Sections representing wild-type (**A–C**) and Stat3 fl/fl;WC mice (**D–F**) were incubated with primary antibodies directed against Npt2b (red) and E-cadherin (green). The localization of specifically bound primary antibodies was detected with fluorescent-conjugated secondary antibodies. In wild-type animals, Npt2b was evident on the apical membrane at lactation day 10 (**A**) but was absent by day 2 involution (**B**) and in the remodeled gland at day 6 involution (**C**). The deletion of Stat3 via Cre-loxP-mediated recombination had no effect on Npt2b protein at day 10 of lactation (**D**). By day 2 of involution, extensive apical Npt2b remained evident in Stat3 fl/fl;WC mice (**E**, white arrowhead) and was further accompanied by the appearance of immunoreactive Npt2b in the lumen of the secretory structures (**E**, yellow arrowhead). At day 6 of involution, Npt2b immunoreactivity was detected only in the lumen of Stat3 fl/fl;WC mice (**F**, yellow arrowheads) and was no longer apparent on the apical membrane. Bar = 100 μm.

of normal cyclin D1 levels in the IKKα mutant mice resulted in the detectable expression of apical Npt2b protein in all alveolar structures (Figure 3D). These data demonstrate that attainment of secretory function on restoration of cyclin D1 levels is associated with the induction of apical Npt2b protein and reestablishment of the secretory cell proteotype.

Proteotypic Analysis of Mammary Epithelium in Mice with a Loss of the Inhibin βB-subunit

The functional disruption of the inhibin βB-subunit in mice results in the birth of pups with open eyelids, and pups born to female inhibin βB knockout mice fail to thrive (Vassalli et al. 1994). A study of the mammary gland phenotype in these mice revealed the presence of abnormal terminal end buds, delayed ductal outgrowth and a failure of the alveolar compartment to develop during pregnancy and lactation (Robinson and Hennighausen 1997). Transplantation experiments further established that the absence of inhibin βB in the stromal compartment, but not the epithelial compartment, was responsible for the phenotype observed. However, despite reduced alveolar development, expression of the milk protein genes encoding WAP, β-casein, WDNM1, and α-lactalbumin was comparable to that of hemizygous and wild-type mice, suggestive of normal differentiation.

To further understand and define the molecular lesions in these mice, we determined the mammary proteotype. Analysis of virgin inhibin βB-null mice revealed the presence of NKCC1 and AQP5 in developing ductal structures similar to that observed in wild-type mice (data not shown). Similarly, AQP5 was absent and NKCC1 protein levels were downregulated during pregnancy in inhibin βB-null and wild-type mice (data not shown). Despite the inability of the mice to lactate, inhibin βB-null mice demonstrated an appropriate induction of Npt2b protein on the apical membrane of developing alveolar cells and the induction of WAP protein at mid-pregnancy (data not shown), and Npt2b was observed at levels comparable to wild-type mice at parturition (cf. Figures 3A and 3E). These data demonstrate that the mammary proteotype in inhibin βB-null mice is normal, and further suggest that the defect in epithelial cell development is not associated with a lack of functional differentiation but may

be a result of reduced proliferation and/or other cellular abnormalities independent of cell differentiation.

Delayed Involution in the Absence of Stat3 Is Associated with a Persistence of Apical Npt2b

Mammary gland involution is associated with a rapid loss of Stat5 phosphorylation and a concomitant increase in Stat1 and Stat3 phosphorylation (Liu et al. 1996). Targeted disruption of the Stat3 gene (Takeda et al. 1997) results in rapid degeneration of the embryos between embryonic days 6.5–7.5, which precludes the analysis of mammary development and function in Stat3-null mice. To overcome the embryonic lethality, mice in which the Stat3 gene was conditionally targeted using the Cre-loxP recombination system were generated (Takeda et al. 1998). These mice have been used to mediate deletion of Stat3 in T-cells (Takeda et al. 1998) and mammary epithelium (Chapman et al. 1999) using tissue-specific Cre recombinase-expressing mice.

We have recently examined (Humphreys et al. 2002) the effect of Cre-mediated deletion in a mouse model containing a conditionally targeted Stat3 gene (Raz et al. 1999). Stat3 fl/fl mice were crossed with WAP-Cre expressing mice (Wagner et al. 1997) to produce Stat3 fl/fl;WC mice, resulting in the deletion of Stat3 (exons 16–21) specifically in the secretory epithelial cell compartment during mid-pregnancy. Whereas Cre-mediated deletion of Stat3 had no detectable effect on mammary gland development or lactational competency before involution, the epithelial compartment failed to undergo apoptosis-induced remodeling after cessation of suckling and retained large alveolar structures, open lumina, and evidence of milk secretion (Humphreys et al. 2002). To further understand the molecular consequences of the defective involution process in these mice, we examined the mammary proteotype. Determination of NKCC1 and AQP5 protein revealed appropriate expression during virgin development and their reduction during pregnancy (data not shown). Npt2b protein was induced during mid-pregnancy and persisted throughout lactation, similar to the pattern observed in wild-type mice (data not shown; and Figures 4A and 4D). Analyses of involution time points in wild-type animals demonstrated that apical Npt2b was lost within 2 days of weaning (Figure 4B) and coincided with the appearance of immunoreactive protein in the lumen (Figure 4B, yellow arrowhead). In contrast, extensive Npt2b protein was present on the apical membrane of alveolar cells at day 2 of involution (Figure 4E, white arrowhead) in Stat3 fl/fl;WC mice, which was accompanied by immunoreactive protein in the lumen (Figure 4E, yellow arrowhead). By day 6 of involution, extensive remodeling of the gland had occurred in wild-type animals and there was no evidence of Npt2b immunostaining

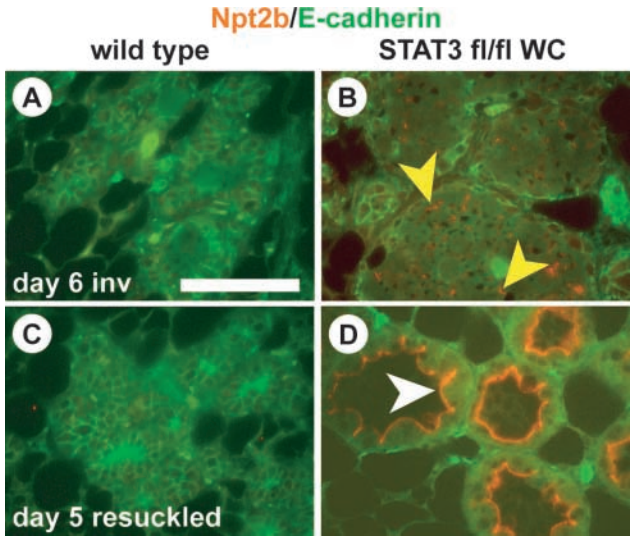

Figure 5 Resuckling results in restoration of apical Npt2b protein in the mammary gland of Stat3 fl/fl;WC mice but not of wild-type mice. Sections representing wild-type and Stat3 fl/fl;WC from day 6 involution (**A,B**) and day 5 resuckled mice (**C,D**) were incubated with primary antibodies directed against Npt2b (red) and E-cadherin (green). The localization of specifically bound primary antibodies was detected with fluorescent-conjugated secondary antibodies. Wild-type mice that had undergone 6 days of involution had no detectable apical Npt2b protein (**A**). In contrast, there was evidence of immunoreactive Npt2b protein in the lumen of day 6 involuted mammary glands of Stat3 fl/fl;WC animals (**B**, yellow arrowheads). Although resuckling of wild-type glands for 5 days failed to stimulate the appearance of apical Npt2b (**C**), significant apical Npt2b was observed in Stat3 fl/fl;WC mice (**D**, white arrowhead). Bar = 100 μm.

(Figure 4C). On the contrary, although apical Npt2b was no longer evident in Stat3-null mice at day 6 of involution (Figure 4F), large alveoli with lumina containing secretory products were apparent and extensive Npt2b immunoreactivity was observed in the lumen, which was often associated with secreted lipid droplets (Figure 4F, yellow arrowhead). The persistence of apical Npt2b protein in Stat3 fl/fl;WC mice during involution supports and extends the observation of a delay in the remodeling of the epithelium after weaning and further demonstrates the use of proteotyping as a means to determine specific developmental lesions (Humphreys et al. 2002).

To broaden these observations and to further establish the lactational competency and mammary proteotype of Stat3 fl/fl;WC glands, we examined the status of Npt2b in Stat3 fl/fl;WC and wild-type mice after 6 days of involution followed by 5 days of re-suckling (Humphreys et al. 2002). In the case of wild-type mice, all pups (12/12) failed to thrive, whereas pups (18/20) placed with Stat3 fl/fl;WC mice did thrive. The ability of the Stat3 fl/fl;WC mice to lactate was associated with evidence of secreting alveolar struc-

tures, increased WAP protein levels, and nuclear accumulation of Stat5 (Humphreys et al. 2002). Although Npt2b was no longer present on the apical membrane in day 6-involuted Stat3 fl/fl;WC mice, considerable immunoreactive protein was evident in the lumen of the alveolar structures (Figure 5B, yellow arrowheads) compared to wild-type mice (Figure 5A). On re-suckling, no Npt2b protein was apparent in wild-type mice (Figure 5C) and the epithelial structures remained undeveloped. However, in Stat3 fl/fl; WC mice, apical Npt2b protein was induced robustly (Figure 5D, white arrowhead) and was present in all alveoli examined (data not shown). These results establish, from a proteotypic perspective, that the rescue of secretory function in Stat3 fl/fl;WC mice coincides with the induction of apical Npt2b protein. These findings not only demonstrate the sustained reversibility of involution in the absence of Stat3 but also further establish the application of proteotyping as a means to assess mammary epithelial cell development.

Discussion

Many mouse models have been generated that exhibit defective mammary gland development and include altered activity of membrane receptors, tyrosine kinases, and transcription factors (Shillingford and Hennighausen 2001). At present, limited techniques are available to examine the developmental lesions in such mouse models. For example, the analysis of milk protein gene expression in mouse models that have a defective PRL signaling pathway is not an ideal readout because this pathway directly affects milk protein gene expression. Furthermore, the analysis of proliferation and apoptosis and the assessment of histology provide only information about overall cell status and gross morphological defects and fails to address underlying molecular lesions. Another limitation is the inability to differentiate between ductal epithelial cells present in virgin mammary tissue and alveolar epithelial cells that develop during mid-pregnancy under the influence of PRL signaling.

Using database screens and immunohistochemistry, we have identified three proteins that are regulated in a development-specific manner and are representative of distinct stages of mammary gland development. In this study, we have used these protein markers as a means to further understand mammary-associated developmental lesions in several mouse models (Cao et al. 2001; Humphreys et al. 2002; Miyoshi et al. 2001; Robinson and Hennighausen 1997; Shillingford et al. 2002b). Using AQP5, we demonstrate that postpartum Stat5- and Jak2-null mammary transplants exhibit significant apical AQP5 protein, indicative of the presence of virgin-like ductal structures rather than secretory structures. This observation supports previous data suggesting the duct-like nature of PRLR-, Stat5-, and Jak2-null mammary epithelium at parturition (Miyoshi et al. 2001; Shillingford et al. 2002b), and further demonstrates the ability of AQP5 to discriminate among virgin ductal cells, ductal cells at pregnancy, and secretory cells at parturition. The analysis of Npt2b protein has yielded novel insights and further understanding of the developmental lesions observed in the mammary epithelium of inhibin βB-null (Robinson and Hennighausen 1997), IKK$\alpha^{AA/AA}$ (Cao et al. 2001), and Stat3 fl/fl;WC mice (Humphreys et al. 2002). It is therefore apparent that the epithelial defect previously described in inhibin-βB-null mice (Robinson and Hennighausen 1997) is not associated with impaired differentiation because Npt2b protein is expressed appropriately and is present at normal levels in postpartum glands, but is probably due to reduced proliferation of the epithelium. These data demonstrate that the analysis of Npt2b protein provides a means to differentiate between developmental lesions associated with proliferation vs differentiation. The analysis of Npt2b protein in IKK$\alpha^{AA/AA}$ mice (Cao et al. 2001) suggests that Npt2b can be further used to assess differentiation defects associated with pregnancy-mediated mammary gland development. Furthermore, the rescue of both the lactation phenotype and Npt2b protein expression in IKK$\alpha^{AA/AA}$ mice by re-establishment of the cyclin D1 levels points to a role for cyclin D1 in mammary epithelial cell differentiation, in addition to its established role in mammary epithelial cell proliferation (Yu et al. 2001). Finally, we were able to assess at the molecular level the developmental lesions connected with delayed involution in Stat3 fl/fl;WC mice (Humphreys et al. 2002) and to demonstrate the maintenance of apical Npt2b and the significantly extended reversibility of the involution process.

The localization, cell specificity, and developmental profile of NKCC1, AQP5, and Npt2b proteins in mammary tissue provide unique insight into the regulation of specialized transporter proteins (Shennan and Peaker 2000) and demonstrate the dynamic nature of their function. Therefore, the presence of AQP5 and high levels of NKCC1 protein in mammary ductal cells of the virgin animal and the induction of Npt2b protein in developing mammary alveolar structures in the pregnant animal suggest distinctive physiological roles for these membrane transporters during the course of normal mammary gland development. In this connection, we have recently shown that disruption of NKCC1 results in increased branching morphogenesis in virgin mammary tissue but does not have any overt effect on alveolar development during pregnancy or milk secretion at lactation (Shillingford et al. 2002a). These observations corroborate the protein data and support the hypothesis that NKCC1 is

involved in ductal cell development in the virgin animal but not in alveolar cell development or function in the pregnant animal. To our knowledge, this study is the first to establish the localization of AQP5 in the apical membrane of virgin mammary ductal epithelial cells. Given that AQP5 is a member of the aquaporin family (Verkman and Mitra 2000), which mediates water and small solute transport across membranes, it would be of physiological interest to determine whether AQP5 plays a role in ductal cell development. Furthermore, on the basis of EST database searches, both AQP5 and NKCC1 are highly expressed in cDNA libraries derived from mammary tumors, suggesting that these proteins may serve as prognostic markers for tumor development and/or progression. On the basis of extensive physiological experiments, it has been suggested that a functional Na–Pi co-transport process exists in the basolateral membrane of rat alveolar cells during lactation (Shillingford et al. 1996). The identification on the apical membrane of an immunoreactive protein corresponding to the intestinal Na–Pi co-transporter isoform Npt2b (Hilfiker et al. 1998) suggests that the apical membrane may also mediate Na–Pi co-transport. However, assessment of true physiological function will have to come from experiments that can demonstrate Na-dependent Pi transport in isolated apical membranes. The induction of apical Npt2b protein during mid-pregnancy suggests that the presence of Npt2b may be important for secretory function. This hypothesis is supported by the observation that the absence of intact IκB kinase function, which results in the inability of mice to lactate, corresponds with a lack of Npt2b induction.

We have demonstrated that the IHC analysis of NKCC1, AQP5 and Npt2b proteins using specific antibodies provides a novel means to examine mammary-specific lesions observed in transgenic and gene knockout mouse models. The identification, localization, and developmental profile of these proteins and their defined application constitute what we refer to as proteotyping: tissue typing based on the analysis of cell type-specific changes in protein levels using IHC techniques. Although we have demonstrated the use of this method as it applies to mammary gland development, we believe that the concept of proteotyping is equally applicable to other tissues. In particular, we propose that proteotyping could be used to define changes in protein expression patterns that are associated with tumorigenesis and could thus serve as a prognostic tool. Such an application would probably involve a multidisciplinary approach, combining micro-array, protein array and tissue array analyses. One of the distinct advantages of using proteotyping is the ability to determine the localization of the protein of interest in specific cell types. For this reason, proteotyping offers a more sensitive method than the determination of changes in whole protein or mRNA levels, which would reflect the contribution of many different cell populations.

In conclusion, our results define the concept of a proteotype and further demonstrate the application of proteotyping in the analysis of developmental lesions observed during mammary epithelial cell development in transgenic and gene knockout mouse models. With the use of the mammary gland as a model system, we suggest that the identification and characterization of other tissue proteotypes using defined protein markers may represent a technique that can be applied to the analysis of normal tissue development and as a prognostic tool in the analysis of tumorigenesis.

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